ORIGINAL ARTICLE INFECTIOUS DISEASES

Simultaneous detection of gastrointestinal pathogens with a multiplex Luminex-based molecular assay in stool samples from diarrhoeic patients

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Abstract

We have evaluated the multiplex molecular method xTAG® Gastrointestinal Panel (GPP) for detecting pathogens in stool samples of diarrhoeic patients. We collected 440 samples from 329 patients (male:female ratio of 1.2:1), including 102 immunosuppressed adults, 50 immunosuppressed children, 56 children attending the neonatal unit and 121 children attending the emergency unit. Of these, 176 samples from 162 patients were xTAG® GPP positive (102 viruses, 61 bacteria and 13 parasites) and the assay was more sensitive than the conventional test for detecting rotavirus ($p < 0.01$), noroviruses ($p < 0.0001$), *Salmonella* spp. ($p < 0.001$), *Campylobacter* spp. ($p < 0.001$) and toxigenic *Clostridium difficile* (p 0.005). The predominant pathogens were viruses (23.2%), with rotavirus (15.9%) being the most common. Bacterial agents were detected in 13.9%; the most common was *Salmonella* spp. (4.8%). Parasites were detected in 2.9%; *Cryptosporidium* spp. (2%) was the most common. There were 31 co-infections (7% of samples), involving two pathogens in 23 (5.2%) and three pathogens in eight (1.8%) samples. There were 113 (92.6%) positive samples from the children attending the emergency unit, 25 (17%) positive samples from immunosuppressed adults, 22 (25.3%) positive samples from immunosuppressed children and 16 (19%) positive samples from children attending the neonatal unit. The low turnaround time and technical hands-on time make this multiplex technique convenient for routine use. Nevertheless, conventional bacterial culture and parasitological stool examination are still required to detect other pathogens in specific cases and to determine susceptibility to antibiotics.

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Introduction

Acute infectious gastroenteritis (AIG) is a major economic burden in developed countries due to the frequent need to hospitalize young children and the elderly [1]. Viruses are the main agents responsible [2] and they often result in outbreaks. Food-borne bacteria are also a serious public health threat worldwide. The Shiga-like toxin-producing *Escherichia coli* (STEC) (shiga toxin 1 and 2 (stx1/stx2)) has recently joined the usual enteric pathogenic bacteria such as *Salmonella* spp., *Shigella* spp. and *Yersinia enterocolitica* [3]. This pathovar may cause severe and potentially fatal illnesses such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS). Bacterial agents are traditionally identified by enrichment culture and biochemical assays, but these techniques are time-consuming. Oligonucleotide-based microarrays using molecular PCR methods have been developed that rapidly detect rRNA [4] and

virulence-factor genes [5] in food, soil and water. These techniques are also sensitive enough to detect the major gastrointestinal bacteria in human stool samples [6]. The major aetiological agents of parasitic diarrhoea are *Cryptosporidium* spp., *Entamoeba histolytica* and *Giardia liamblia*. Molecular techniques that are more sensitive and specific than microscopy and antigen detection are also increasingly available [7].

It is difficult to differentiate among viral, bacterial and parasitic aetiological agents because the symptoms are so similar. In fact, 80% of all cases of diarrhoea are presently unidentified [8], and antibiotics are often used inappropriately. New molecular techniques can detect bacteria [9], viruses [10,11] or parasites [7–12] independently or simultaneously [13]. We have used the xTAG® Gastrointestinal Panel (Luminex Molecular Diagnostics, Toronto, ON, Canada) to detect and identify the majority of microbiological agents of AIG. The results were compared with those obtained by routine methods. We also analysed the spread of pathogens among four populations of patients presenting with AIG: immunosuppressed adults, immunosuppressed children, children attending the neonatal unit and children attending the emergency unit.

Materials and Methods

Sample collection

A total of 440 stool samples prospectively collected from 329 diarrhoeic patients attending the Toulouse University Hospital from February to December 2011 were sent to the Department of Microbiology for investigation. Stool samples were collected into empty sterile flasks and anorectal swabs were placed in transport medium (TGV anaerobic, Bio-Rad, Hercules, CA, USA) for bacterial culture and in virologically suitable Virocult® (Kitvia, Labarthe Inard, France) for virological investigation.

Routine diagnostic methods

Rotavirus-adenovirus and noroviruses were detected directly in stool samples with rapid immunochromatographic tests: COMBO ROTA ADENO+ STICK (All-Diag, Strasbourg, France) and Immunocard Norovirus (Meridian Bioscience, Cincinnati, OH, USA). Minimum essential medium (2 mL) was then added to each stool sample. These samples were freeze-thawed to disrupt cells, centrifuged, and the supernatants saved. Bacteria were cultured from whole stool and anorectal swabs on each medium plate and selenite broth. Classic enteric pathogens (*Salmonella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, *Shigella* spp., *Vibrio cholerae,* toxigenic *Clostridium difficile,* pathogenic *Escherichia coli*) were detected by standard culture methods. Suspicious colonies were identified using microscopic and biochemical tests. *Salmonella* serovars were specified by O and H antigen serotyping. Toxigenic *Clostridium difficile* was identified by immunochromatographic assay (Immunocard ICTAB, Meridian Bioscience), which identifies A and B toxins. The virulence genes of toxigenic *Escherichia coli* were detected by multiplex PCR (Genotype EHEC, Hain LifeScience, Nehren, Germany) from Enterobacteria colonies [14]. Different protocols were used to analyse the stools of children and adults. We looked for toxigenic *Clostridium difficile* and *Escherichia coli* pathovars in all the samples from children, but only in those from certain adults: post-antibiotherapy diarrhoea and nosocomial outbreaks for toxigenic *Clostridium difficile*, or an epidemiological infection for STEC. A routine stool culture usually took 72 h or longer to complete.

We looked for protozoa (*Entamoeba histolytica* and *Giardia liamblia*) by microscopic examination of fresh stools and of samples fixed/concentrated with merthiolate-iodine-formaldehyde (MIF). *Cryptosporidium* spp. was detected by a modified Zielh–Neelsen and auramine staining method [15].

Molecular diagnostic assay

Total nucleic acids were extracted from the stool supernatants with the MagNA Pure 96™ DNA and Viral NA Small Volume Kit (Roche Molecular Diagnosis, Meylan, France) on the MagNA Pure 96. Input and output volumes were 200 and 100 μL.

The multiplex xTAG® Gastrointestinal Panel was used to detect 15 microbiological gastrointestinal pathogens or toxins: rotavirus A, adenoviruses 40 and 41, noroviruses GI and GII, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Clostridium difficile* toxin A/toxin B, STEC, Enterotoxinogenic *Escherichia coli* heat stable and heat labile toxin (ST/LT) (ETEC), enterohaemorragic *Escherichia coli* O157 (EHEC), *Yersinia enterocolitica*, *Vibrio cholerae*, *Giardia liamblia*, *Cryptosporidium* spp. and *Entamoeba histolytica*. Multiplex RT-PCR was performed on a GeneAmp 9700 ABI thermocycler (Applied Biosystems, Santa Clara, CA, USA) using 10 μL of nucleic acid. Biotin-tagged amplicons were generated if a pathogen was present. Anti-tags coupled to colour-coded beads generated a bead-amplicon complex subsequently detected on the Luminex200 reading system. An internal control (bacteriophage MS2) was included in each specimen. Raw data were analysed with the TDAS GPP version 1.11 (xTAG Data Analysis Software): positive and negative results were linked to a ratio between the target median fluorescence intensity (MFI) and the threshold. Samples showing discrepant adenovirus results were tested on the Light Cycler 2.0™ using an in-house real-time PCR [16].

Statistics

Data were analyzed using StataTM software (StataCorp, Houston, TX, USA). The match between the assays was

assessed using the Mc Nemar chi-squared test. p-values of <0.05 were considered significant.

Results

Four hundred and forty stool samples were prospectively collected from 329 diarrhoeic patients (male:female ratio, 1.2:1). Three groups included immunosuppressed hospitalized patients: 102 adult organ transplant recipients (mean age, 50.6; median, 56; range, 17–75), 50 immunosuppressed children (mean age, 5; median, 7; range, 0–14) and 56 children attending the neonatal unit (aged under 1 year). The 121 children attending the emergency unit (mean age, 2.80; median, 9; range, 0–16) were considered to be outpatients.

Pathogens detected with the xTAG® GPP

We found 176 (40%) positive samples collected from 162 patients (Fig. 1). Of these, 102 samples were positive for viruses, with rotavirus being the most common (70 samples; 15.9%), followed by norovirus (30 samples; 6.8%). Adenoviruses were detected in two samples. Bacterial agents were detected in 61 (13.9%) samples. *Salmonella* spp. was the most common (21 samples; 4.8%), followed by toxigenic *Clostridium difficile* (18 samples; 4.1%) and *Campylobacter* spp. (13 samples; 2.9%). STEC was detected in seven (1.6%) samples. One sample was positive for *Shigella* spp. and one was positive for the O157 *Escherichia coli* serovar. Parasites were detected in 13 (2.9%) samples, with *Cryptosporidium* spp. being the most common (nine samples, 2%). Three samples were positive for *Entamoeba histolytica* and one for *Giardia liamblia*.

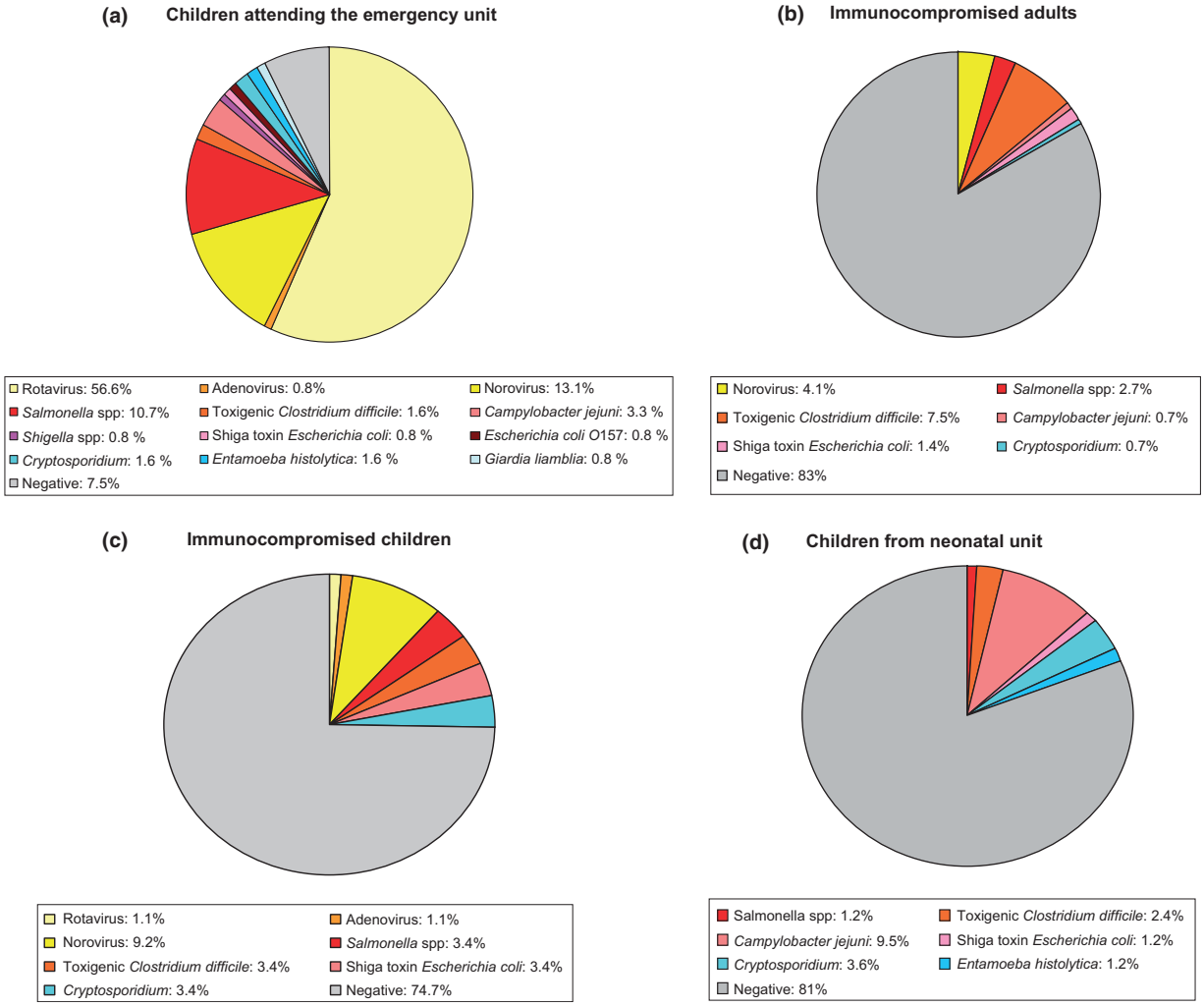

FIG. 1. (a) Pathogens in 113 samples collected from the 121 children (21 co-infections). (b) Pathogens ($n = 25$) detected among the 102 adult immunocompromised patients attending the emergency unit (three co-infections). (c) Pathogens ($n = 22$) detected among the 50 immunocompromised children (four co-infections). (d) Pathogens ($n = 16$) detected among the 56 children attending the neonatal unit (three co-infections).

There were 31 co-infections (7% of samples) of viruses and/or bacteria and/or parasites (Table 1). Co-infections involved two pathogens in 23 (5.2%) samples and three pathogens in eight (1.8%) samples.

Comparison of the xTAG® GPP and conventional detection

The two methods were compared using the data from samples tested by both techniques (Table 2).

Virological results. The xTAG® GPP detected more viruses than did conventional methods: for norovirus, 6.8% (30/440 samples) were positive compared with 0.3% (1/287 sample) using conventional testing ($p < 0.0001$); for rotavirus, 15.9% (70/440 samples) were positive compared with 14.4% (63/438 samples) using the conventional detection ($p < 0.01$). For adenovirus, 0.4% (2/440 samples) were positive using the xTAG® GPP and negative using conventional technique.

The immunochromatographic technique detected norovirus in one sample, rotavirus in two samples and adenovirus in five samples that were negative with the xTAG® GPP.

Samples giving discordant adenovirus results were tested with the in-house real-time PCR: this gave results similar to those of xTAG® GPP.

Bacteriological results. The xTAG® GPP identified 61 (13.9%) samples that contained bacteria, while conventional methods identified only 21. Toxigenic *Clostridium difficile* was detected in 18/440 samples (4.1%) by xTAG® GPP, and in 1.3% (5/372 samples; p 0.005) by conventional methods. The corresponding figures for *Salmonella* spp. were 21/440 (4.8%) and 9/413 (2.2%; $p < 0.001$); for *Campylobacter* spp. they were 13/440 (2.9%) and 1/415 (0.2%; $p < 0.001$), and for STEC they were 7/440 (1.6%) and 5/273 (1.8%; ns). Both techniques detected one sample containing *Shigella* spp., while the xTAG® GPP detected one with *Escherichia coli* O157 serovar. Neither technique detected any samples containing ETEC, *Yersinia enterocolitica* or *Vibrio cholerae*.

The conventional technique detected *Salmonella* spp. in two samples that were negative with the xTAG® GPP.

Parasitological results. The xTAG® GPP detected parasites in 13 stool samples; only one of these was tested with a conventional technique and it was negative. *Cryptosporidium* spp. was found in nine samples, *Entamoeba histolytica* in three samples and *Giardia liamblia* in one sample. The samples that were positive for *Entamoeba histolytica* gave an MFI value close to the threshold.

Overall, the xTAG® GPP system identified 34 samples that tested negative for pathogens and the internal control (uninterpretable results). These samples were either negative or not tested by routine techniques.

TABLE 1. Pathogens detected in co-infections (CoIs)

Pathogens	*Rotavirus*	*Norovirus*	*Adenovirus*	*Campylobacter* spp.	*Salmonella* spp.	*Shigella* spp.	STEC	Toxigenic *Clostridium difficile*	*E. coli O157*	*Cryptosporidium* spp.	*Giardia liamblia*	*Entamoeba histolytica*	No. of samples with CoIs
No. of single infections	54	11	1	10	8	1	3	15	0	1	1	1	106
No. of CoIs	16	19	1	3	13	0	4	3	1	8	0	2	70
Total	70	30	2	13	21	1	7	18	1	9	1	3	176
	O		O										1
	O			O									2
	O				O								1
	O				O				O				1
	O	O											6
	O	O			O					O			3
	O	O						O					1
	O	O					O+						1
	O+	O			*+								3
	*+									O+			2
	O+	O			+					+			1
	+						O			O			1
	O			¤	*			¤		¤			1
					¤					*			1
					O			¤				O	2
							¤	¤		O			1
							O						1

'O' represents children attending the emergency unit; '+' represents immunocompromised adults; '*' represents immunocompromised children; '¤' represents children attending the neonatal unit.

TABLE 2. Results of the xTAG®-GPP assay and conventional detection.

		Conventional routine detection				
Pathogen	**Routine test**	**Positive (*N*)**	**Negative (*N*)**	**Not done (*N*)**	**Total**	**Mc Nemar chi-squared test: p**
xTAG results Rotavirus						
Positive	COMBO ROTA ADENO+ STICK	61	9	0	70	0.008
Negative		2	332	2	336	
No call		0	34	0	34	
Total		63	375	2	440	
Norovirus GI and GII						
Positive	Immunocard Norovirus	0	17	13	30	<0.0001
Negative		1	250	125	376	
No call		0	19	15	34	
Total		1	286	153	440	
Adenovirus 40 and 41						
Positive	COMBO ROTA ADENO+ STICK	0	2	0	2	ns
Negative		5	396	3	404	
No call		0	34	0	34	
Total		5	432	3	440	
Campylobacter spp.						
Positive	Standard culture	1	12	0	13	<0.001
Negative		0	368	25	393	
No call		0	34	0	34	
Total		1	414	25	440	
Salmonella spp.						
Positive	Standard culture	7	14	0	21	<0.001
Negative		2	356	27	385	
No call		0	34	0	34	
Total		9	404	27	440	
Shigella spp.						
Positive	Standard culture	1	0	0	1	ns
Negative		0	378	27	405	
No call		0	34	0	34	
Total		1	412	27	440	
Toxigenic *Clostridium difficile toxin A and B*						
Positive	PCR	5	8	5	18	0.005
Negative		0	331	57	388	
No call		0	28	6	34	
Total		5	367	68	440	
Shiga-like toxin producing *Escherichia coli* shiga (toxin 1 and 2 (stx1/stx2) (STEC)						
Positive	PCR	5	2	0	7	ns
Negative		0	258	141	399	
No call		0	8	26	34	
Total		5	268	167	440	
Enterotoxigenic *Escherichia coli* heat stable and heat labile toxin (ETEC)						
Positive	PCR	0	0	0	0	
Negative		0	266	140	406	
No call		0	8	26	34	
Total		0	274	166	440	
Escherichia coli O157						
Positive	PCR	0	1	0	1	
Negative		0	265	140	405	
No call		0	8	26	34	
Total		0	274	166	440	
Yersinia enterocolitica						
Positive	Standard culture	0	0	0	0	
Negative		0	379	27	406	
No call		0	34	0	34	
Total		0	413	27	440	
Vibrio cholerae						
Positive	Standard culture	0	0	0	0	
Negative		0	379	27	406	
No call		0	34	0	34	
Total		0	413	27	440	
Giardia liamblia						
Positive	Microscopic examination	0	0	1	1	
Negative		0	118	287	405	
No call		0	8	26	34	
Total		0	126	314	440	
Cryptosporidium spp.						
Positive	Microscopic examination	0	1	8	9	
Negative		0	117	280	397	
No call		0	8	26	34	
Total		0	126	314	440	
Entamoeba histolytica						
Positive	Microscopic examination	0	0	3	3	
Negative		0	118	285	403	
No call		0	8	26	34	
Total		0	126	314	440	

ns, not significant; no call, uninterpretable results.

Epidemiological features

Most of the samples (113/122; 92.6%) collected from the children attending the emergency unit tested positive, while there were fewer positive samples among those collected from immunosuppressed adults (25/147; 17%), from immunosuppressed children (22/87; 25.3%),

and from children attending the neonatal unit (16/84; 19%) (Fig. 1a–d).

Nevertheless, the distribution of the pathogens differed from one patient population to another; 98.6% (69/70) of rotavirus and 53.3% (16/30) of norovirus were detected in the samples from children attending the emergency unit. Norovirus was also detected in 26.7% of the samples (8/30) from immunocompromised children and in 20% of those from adults (6/30). *Salmonella* spp. was detected most frequently in samples from children attending the emergency unit (13/21; 61.9%), while toxigenic *Clostridium difficile* was most common in those from immunocompromised adults (11/18; 61.1%).

Discussion

Four hundred and forty stool samples collected from 329 diarrhoeal patients were analysed to evaluate the performance of the xTAG® GPP. The rates of detection were compared with those obtained with routine diagnosis techniques. We also determined the prevalence of pathogens in four populations of patients.

The multiplex PCR technique was statistically more sensitive than conventional techniques and allowed the aetiological diagnosis of some cases of gastroenteritis whose origin had been previously unknown. This was especially the case for norovirus, *Salmonella* spp., *Campylobacter* spp. and *Cryptosporidium* spp. This is comparable with the results of the large longitudinal study by Tam *et al.* [17] conducted in the United Kingdom: norovirus was the most common organism detected and *Campylobacter* spp. the most common bacterium. Novel diagnostic techniques have led to a new appreciation of the impact of noroviruses on AIG. They can be responsible for about 90% of previously undetermined outbreaks of gastroenteritis and 5–30% of cases of sporadic childhood and adult gastroenteritis [17–19]. Norovirus has now spread to healthcare environments worldwide, including both immunocompetent and immunocompromised children and adults [20]. These published data are similar to our results; we detected noroviruses in children attending the emergency unit, and also in immunocompromised adults and children.

The xTAG® GPP did not detect rotavirus in two samples, adenovirus in five samples and norovirus in one sample that were positive with the immunochromatographic tests. These manual techniques include steps that might be dependent on the technician. Positive adenovirus samples were tested with a second molecular test that gave results similar to those of the xTAG® GPP. Molecular techniques are likely to be highly specific as they use specific primers and probes. These results show that immunochromatographic tests can give false-positive results and do not mean that the xTAG® GPP was less sensitive.

The xTAG® GPP detected more bacteria than did conventional methods. Only two samples showed discrepant results for *Salmonella* spp. (positive by standard culture and negative with the xTAG® GPP). The samples in question were positive for *Salmonella enteritidis* and *Salmonella typhi murium*. These pathogens have been detected in other samples that were positive by both techniques, indicating that the xTAG® GPP was well designed to detect them. These discrepant results were probably due to stochastic detection of the pathogens in these samples. These results are similar to those of a previous study using the Seeplex® diarrhoea ACE detection system [13]: three samples were not detected by the molecular test.

We routinely tested samples from children for toxigenic *Clostridium difficile* because numerous surveys and studies looking at the significance of isolating this pathogen have demonstrated its pathogenic potential in children [21,22]. Cancer chemotherapy also seems to be as important as antibiotics in *Clostridium difficile* infection in paediatric inpatients and outpatients.

One sample that tested negative for shigatoxin genes was positive for the *Escherichia coli* O157 serovar, indicating that the serotype O157 is not necessarily related to the virulence of *Escherichia coli* [14]. The seven samples that tested positive for shigatoxins genes were negative for O157 serovar, indicating that STEC belong to serovars other than O157. Neither ETEC nor *Yersinia enterocolitica* were detected, which is well in accordance with the epidemiological spread of these bacteria: *ETEC* is a gastrointestinal pathogen in developing countries [23] and there is evidence that the spread of *Yersinia enterocolitica* is decreasing [24].

Studies comparing PCR with microscopic examination for protozoa have shown that conventional techniques lack sensitivity. However, we tested only a few samples by conventional methods, making the results difficult to interpret. *Entamoeba histolytica* was detected in stool samples of one child attending the neonatal unit and in those of two children attending the emergency unit. All three gave a slightly positive signal. These results are difficult to interpret in the absence of a reference technique, and might reflect false-positive reactivity.

The xTAG® GPP method efficiently detected co-infections and the results are in agreement with those of previously published studies that detected rates of co-infections of 0.9% (0.02% by conventional methods) [25] and 6.8% (0% by conventional methods) [26]. Other studies on patients with AIG requiring hospitalization have shown very high rates of co-infection (up to 22%) [27,28].

The present study provides a clear picture of the relative frequencies of microbiological pathogens in different patient

populations. Children attending the emergency unit were more frequently infected with a wider diversity of pathogens than were the three other hospitalized populations. Rotavirus, norovirus and *Salmonella* spp. were detected mainly in these children. This is not surprising as the children's emergency unit takes in children presenting with AIG requiring a medical examination. Similarly, in adults, Jansen *et al.* [28] detected *Campylobacter* spp. (35%) most frequently, but also norovirus (23%), *Salmonella* spp. (20%) and rotavirus (15%). *Cryptosporidium* spp. was mainly detected in children <5 years old: this is similar to results reported by Yoder *et al.* [29] in the United States.

Most of the samples from the other three hospitalized groups of patients were negative: toxigenic *Clostridium difficile* was the most prominent pathogen in those that were positive. This is comparable to the results of Raines, who showed that this pathogen is a major cause of AIG in non-HIV immunosuppressed patients [30]. No viruses were detected in samples from children attending the neonatal unit. The AIG of most of the hospitalized patients was due to factors other than infections, for example the therapy itself in transplant recipients.

This new technique dramatically shortens turnaround time: approximately 4 h are needed to provide the results for 15 pathogens. Conventional techniques require at least 2 days, involve many and various technical steps and need a range of technical competences (culture, PCR and microscopy). Like Taniucchi *et al.* [9] we have clearly shown that total nucleic acid extracted directly from stool samples can be used for molecular diagnosis; all microbiological pathogens can be detected in a single test, leading to a dramatic decrease of hands-on time. The xTAG® GPP reagents are relatively expensive, but certainly cheaper than the total cost of all the various reagents and the technicians' time required to obtain the same performance. However, this was not evaluated precisely as it was not the aim of the study.

The major limitation of the present study is that some samples were not tested with all the techniques. This may induce a bias, especially in the interpretation of the parasitological results. However, analysis of the available results shows that this new technique was more sensitive than the conventional techniques and that less time was required to obtain similar results.

In conclusion, the multiplex-PCR luminex bead assay is a very sensitive and convenient method for detecting multiple gastrointestinal microbiological pathogens from a single stool sample and may be easily used in routine daily practice. However, some pathogens responsible for food toxi-infections are not detected, including the enteropathogen *Escherichia coli* (EPEC), *Clostridium perfringes*, the enteropathogen *Staphylococcus aureus*, and toxigenic *Aeromonas*. We propose the following workflow pattern. Step one: use the xTAG®-GPP to obtain results for viruses and to identify the main bacterial and parasitological pathogens. Step two: set up bacterial cultures, depending on the results obtained, to determine the antibiotic susceptibility of xTAG®-GPP-positive samples or to identify other bacteria in xTAG®-GPP-negative samples. Stool samples should only be examined for parasites in specific clinical and epidemiological contexts that might involve parasites not detected by the xTAG®-GPP technique.

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Transparency Declaration

All authors declare no conflicts of interest.

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